STUDIES ON THE BLOOD PROTEINS, III

ALBUMIN-GLOBULIN RATIO IN ANTITOXIC IMMUNITY

K. F. Meyer, S. H. Hurwitz, and L. Taussig

From The George Williams Hooper Foundation for Medical Research of the University of California Medical School, San Francisco

INTRODUCTION

In a previous communication,[1] we presented the results of some experimental observations bearing on the relationship between the globulin content of an animal's serum and the grade of immunity developed in it by immunization with various bacteria. These observations brought out several interesting points concerning the relation of the serum globulins to the antibodies, and especially emphasized the lack of parallelism between the globulin content of the blood and the concentration of antibodies. During the past year, we have directed our efforts to a consideration of this problem in its bearing on antitoxic immunity. This subject possesses more than academic interest because of the constant association of the antisubstance with the serum globulin, and because of the practical application that has been made of this knowledge in the preparation and concentration of antitoxic serums.

A restudy of this problem by improved methods was thought desirable because only a few investigations have been made of the globulin variations in relation to potency, and these, for reasons which will be given, are somewhat incomplete, and, therefore, leave certain of the conclusions derived open to doubt.

Brodie[2] was among the first to show that diphtheria antitoxin was completely precipitated from a solution by any means which removed the globulins, and his observations were extended by Belfanti and Carbone[3] and by Seng.[4] But the first notable advance in this study was made by Hiss and Atkinson[5] who estimated the globulin content of the serum of a large number of horses at different stages of immunization against diphtheria toxin. As a result of

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[1] Hurwitz, S. H., and Meyer, K. F.: Jour. Exper. Med., 1916, 24, p. 515.
[2] Jour. Path. and Bacteriol., 1896, 4, p. 460.
[3] Abstr., Centralbl. f. Bakteriol., 1, 1898, 23, p. 906.
[4] Ztschr. f. Hyg. u. Infektionskrankh., 1899, 31, p. 513.
[5] Jour. Exper. Med., 1900, 5, p. 47.

these experiments, these workers arrived at the conservative conclusion that a low potency coincided with a low globulin content, but that it was not possible to regard the absolute amount of globulin as an index of the antitoxin content of the serum. Similar studies were made by Ledingham[6] and by Gibson and Banzhaf.[7] The former concludes from the data furnished by the immunization of a horse and a goat with diphtheria toxin that there would seem to be "some intimate relation between the amount of antitoxin developed and the quantity of the globulins." Gibson and Banzhaf are still more guarded in their conclusions. These workers state that while the greatest rise in the serum globulin was usually coincident with maximum antitoxic potency, the extent of this increase was practically independent of the antitoxin potency when the results on more than 1 horse were contrasted. They, therefore, are inclined to the view that there may be no relation between the absolute or percentage increase of the serum globulin and the antitoxic potency in the plasma of different horses; indeed, they have shown that the increase in the serum globulin of refractory horses may surpass that in the plasma of some of those yielding a high antitoxin. Similar deductions were made by Banzhaf and Famulener[8] in the immunization of goats. They found that the total protein content and the protein partition may be normal at a time when the animal shows the maximum number of antitoxic units.

From our own experiments, we can subscribe to the view that the globulin change is not a necessary concomitant of the elaboration of antibodies, although it is now well established that this protein fraction may increase strikingly during the process of immunization. Our conclusions have been derived from parallel studies of the serum protein fractions and of the antitoxin content of a number of different animals immunized not only with diphtheria toxin, but also with the soluble toxins of the bacilli of tetanus and of botulism. In general, the results obtained in the different animals and with the different soluble toxins point in the same direction and lend support to our previous findings in animals immunized with bacteria and their toxins, namely, that the curves of serum globulin increase and the development of antitoxin potency do not run parallel. Our observations, furthermore, furnish additional evidence that there is some relation between the shock and intoxication caused by the injection of the toxin and the rise in globulins, although we have found an interesting deviation from this reaction in the case of the toxin of the bacillus of botulism. The details of these latter experiments will be given in their proper place, but it may be well to state here that with this particular toxin the immediate result of the inoculation is an increase not of the globulin, but of the albumin fraction. In those instances, however, in

[6] Jour. Hyg., 1907, 7, p. 65.
[7] Jour. Exper. Med., 1910, 12, p. 411.
[8] Collect. Stud. Research. Lab., Dept. Health, N. Y., 1915, 8, p. 208.

which a rise in globulins was found to occur, the increase usually took place at the expense of the albumin fraction, so that the effects of bacterial and soluble toxins are also not unlike in this respect.

METHODS

The experiments were carried out on the horse, dog, goat, and rabbit. The use of these different types of animals is desirable in such a study because, as von Behring has shown, the various animals differ markedly in susceptibility to toxin injections and, therefore, in their degree of response, the dog being the most susceptible and the horse and goat the least susceptible animals. Rabbits do not develop much immunity, but their use served to control our studies on bacterial infection and immunity in which the rabbit was used to the exclusion of all other animals. With the exception of the horse, the experimental animals were kept under constant conditions of diet and activity. The goats were permitted to graze all day, specimens of blood for the protein determinations being collected before the animals were turned out in the pasture. The horses were kept and immunized by the Cutter Laboratory, Berkeley, Calif., and it is a pleasure to acknowledge our indebtedness to Dr. Harry Foster of this laboratory for supplying us regularly with the specimens of blood tested and for many other favors in connection with this work.

For some of the toxins used—diphtheria, tetanus, and botulism—we are indebted to Dr. Foster, to Dr. Krumwiede and Dr. Banzhaf of the Health Dep't of New York City, and to Dr. Dickson of Leland Stanford University.

All of the animals, regardless of type, were immunized with toxin of a known M. L. D. content. The goats were immunized with small but gradually increasing doses of toxin, the amounts chosen varying slightly from those given by Ledingham.[6] A similar procedure applied to dogs soon had to be abandoned because the animals became severely intoxicated and died. Attempts to immunize them with balanced mixtures were successful only when the animals were permitted to recuperate completely from the local and general reactions. In the 2 successful experiments, about 1 month had to elapse between the inoculation of 0.5 c.c. toxin and the 2nd injection of the same amount. The production of a basic immunity in the dog is, therefore, difficult, but, when once attained, amounts of toxin equal to double the initial dosage may be administered subcutaneously in 3-day intervals and no harm be done. We cannot, therefore, agree with Wernicke[9] that dogs are readily immunized, at least when highly potent diphtheria toxin is employed, such as is used at present for the production of antitoxin in the horse.

The rabbits were immunized subcutaneously with toxins which had been attenuated with Lugol's solution, according to the methods recommended by Roux and now used at the Pasteur Institute. In the employment of tetanus, our experiments were successful only after we had diluted the toxin in the proportions suggested by Roux, and permitted the detoxification to continue for at least from one half to three quarters of an hour. Shorter periods of detoxification produced a toxin mixture which caused symptoms of tetanus after a fairly long incubation period.

The antitoxin content of the serums of the experimental animals was determined by several methods. At first, the method commonly employed in North America was used,[10] and a few tests with the Römer intracutaneous

[9] Ztschr. f. Hyg. u. Infektionskrankh., 1892, 28, p. 43.
[10] Bull. 21, Hyg. Lab., U. S. P. H. and M.-H. S., 1902.

method[11] were made. However, Zingher's modification[12] of the intracutaneous test was used for the greater part of the determinations. This method has proved to be reliable and highly satisfactory for experimental work, more particularly for the determination of an antitoxin content below 1 unit. The results of this procedure were further checked by the United States Standard Method, for this could be done economically and in a relatively short time, since we were fortunate enough to obtain well tested L^+ toxins at regular intervals from the Cutter Laboratory.

The potency of the antitetanic serums was roughly estimated by the American method.[13] Properly standardized L^+ toxin was obtained from the Cutter Laboratory. For the determination of the antitoxin content of the dogs immunized with botulism toxin, we chose arbitrarily an amount of toxin (1:800-1:1500) which killed a guinea-pig, weighing 250-350 gm., in 48 hours. Analogous to the standardization of tetanus antitoxin, that amount of antiserum was considered to possess one tenth of an immunity unit which could save the life of a guinea-pig, weighing 250-350 gm., in 48 hours. This, to be sure, is a very rough method, but the only one possible, because of the unavailability of a standard serum.

All the determinations of the albumin, globulin, and nonprotein fractions were made by the microrefractometric method of Robertson.[14]

I. IMMUNIZATION WITH DIPHTHERIA TOXIN

Because of the practical bearing of the relation of the immune bodies to the globulins in the concentration of diphtheria antitoxin from the horse, our attention was first directed to some experimental observations on this animal. From the analytic studies of a number of workers,[15] it has been determined that in the horse there is normally present a higher percentage of globulin in proportion to albumin. According to these observations and our own, the globulins constitute about 50% of the total proteins, giving the protein quotient a value of approximately 1 (Table 1). These studies were then extended to include the goat, dog, and rabbit. In the case of the latter 2 animals, it has been shown in previous communications that the normal protein quotient is

TABLE 1

SERUM PROTEINS OF NORMAL HORSES

Date	Horse	Total Protein, %	Total Albumin, %	Total Globulin, %	Albumin of Total Protein, %	Globulin of Total Protein, %	Non-protein Constituents, %	Protein Quotient
Jan. 6	1	5.8	3.3	2.5	57	43	1.5	1.3
Jan. 6	2	6.6	3.9	2.7	59	41	1.4	1.4
Jan. 13	115	7.4	3.7	3.7	50	50	1.2	1
Average......	....	6.6	3.6	2.9	55	44	1.4	1.2
R. M. Jewett*	....	7.5	4	3.5	53	47	1.65	1.1

* Jour. Biol. Chem., 1916, 25, p. 21.

11 Ztschr. f. Immuitätsforsch., 1909, 3, p. 49.

12 Jour. Infect. Dis., 1916, 19, p. 557.

13 Bull. 43, Hyg. Lab., U. S. P. H. and M. H. S., 1908.

14 Jour. Biol. Chem., 1915, 22, p. 223.

15 Jewett, R. M.: Jour. Biol. Chem., 1916, 25, p. 21.

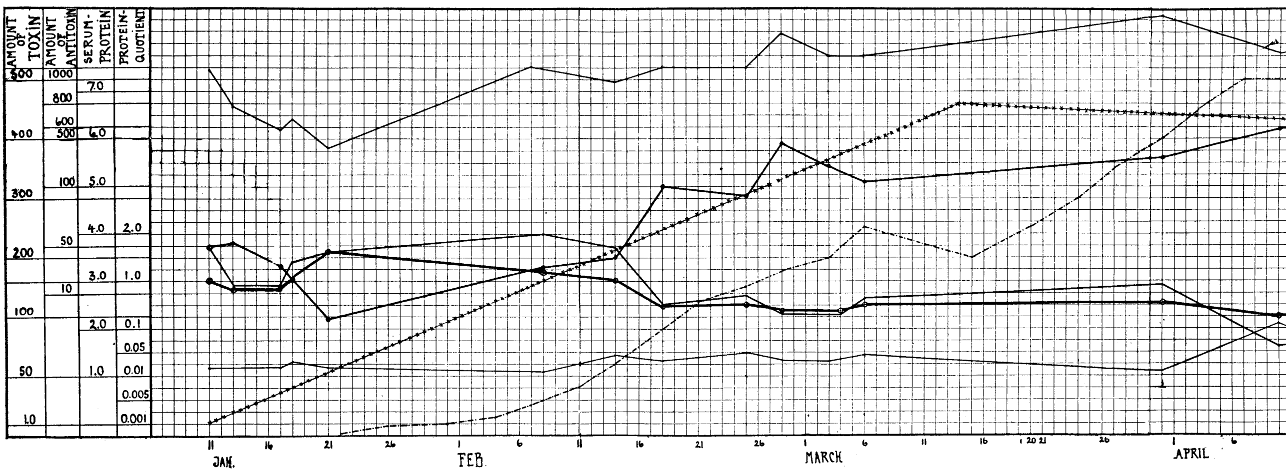
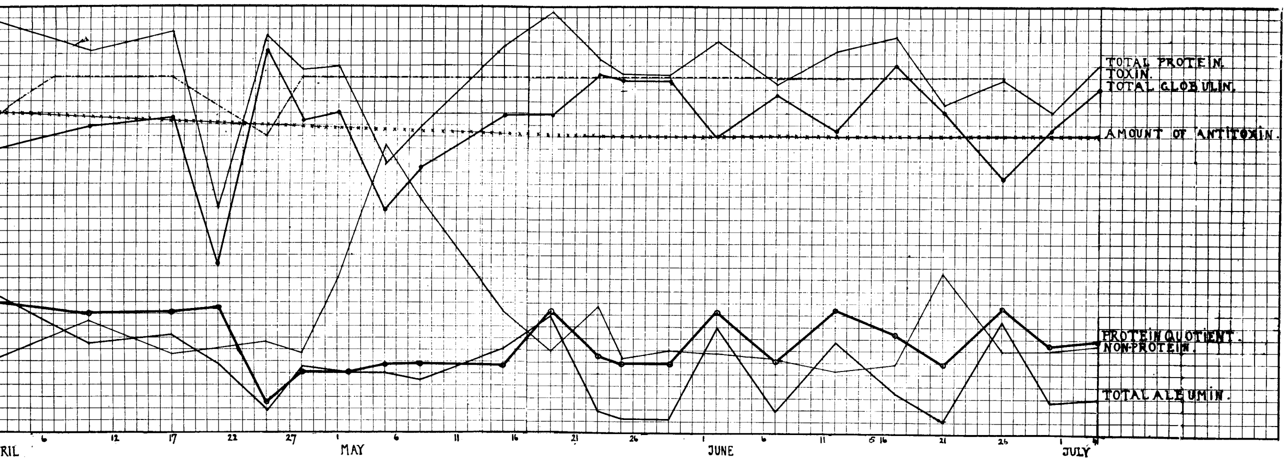


Chart 1.—Horse injected with



jected with diphtheria toxin.

much higher than in the horse (see Table 2); whereas the protein quotient of the goat has been found to be closer to that of the horse. It is well to bear in mind, however, that fluctuations may occur even in normal animals, and it is well, therefore, in all instances to make some preliminary observations on the normal animal before proceeding to the inoculation experiments.

TABLE 2

PERCENTAGES OF SERUM PROTEINS IN NORMAL RABBITS

Rabbit	Total Protein, %	Total Albumin, %	Total Globulin, %	Albumin of Total Protein, %	Globulin of Total Protein, %	Nonprotein Constituents, %	Protein Quotient
327	5.3	4	1.3	75	25	2.1	3
436	6.1	5.2	0.9	85	15	1.3	5.6
449	7.6	5.3	2.3	69	31	1.1	2.2
458	6.3	5.5	0.8	87	13	1.2	6.7
541	6.7	4.8	1.9	71	29	1.2	2.4
570	7	5.2	1.8	74	26	1.3	2.8
571	5.8	3.7	2.1	64	36	1.2	1.7
575	6	4.8	1.2	80	20	1.3	4
576	7	5	2	71	29	1.1	2.4
577	6.6	5.3	1.3	80	20	1.2	4
579	7.3	5	2.3	68	32	1.1	2
580	6.6	5.5	1.1	83	17	1.2	4.9
588	5.8	5	0.8	86	14	1.3	6.1
604	5.3	4.2	1.1	80	20	1.4	4
619	6.7	4	2.7	60	40	1.5	1.5
626	5	3.7	1.3	74	26	1.6	2.8
680	5.8	3.2	2.6	60	40	1.7	1.5
681	5.1	3.1	2	60	40	1.6	1.5
700	5.9	3.9	2	66	44	1	1.5
712	6.1	3	3.1	50	50	1.4	1
713	6.1	3.3	2.8	54	46	1.2	1.2
714	5	2.4	2.6	50	50	1.9	1
715	5.7	3.9	1.8	68	32	1.7	2.1
720	6.1	4	2.1	70	30	1.4	2.3
Average	6.1	4.2	1.8	70	30	1.3	2.8

A. *Immunization of the Horse.* One complete experiment was carried out over a period of 7 months. The detailed observations of this complete study are given in Table 3 and Chart 1. A few points, however, may deserve additional emphasis. At the beginning of the experiment (January 11), the protein quotient was found to be 1, and rose to its highest point, 1.6, 10 days later. The 1st depression in the quotient (rise in globulins) occurred about 4 weeks later, following 8 injections (125 c.c.) of a toxin with a toxicity of 200 M. L. D. Following the repeated injections of large amounts of toxin, a considerable grade of immunity was obtained. A determination of the antitoxic value, on March 14, showed the presence of 800 units. At this period, the protein quotient was about 0.5, and apparently continued unchanged, notwithstanding the repeated injection of larger doses of toxin. The globulins continued to rise, reaching their highest value on April 25, at a time when the animal's hyperimmunity was beginning to diminish, as was evidenced by a reduction in the number of antitoxic units. At this period, it will be noted, from Table 3 and Chart 1, that the globulins constituted 99.6% of the total proteins. Despite the fact that the number of antitoxin units diminished still further toward the end of the experiment, the globulin percentage nevertheless remained almost at a maximum point until the end of our observations. Some fluctuations were encountered, as will be clearly seen in the chart, but these do not appear to have any definite relationship to the periods of inoculation.

TABLE 3

EFFECT OF THE INJECTION OF DIPHTHERIA TOXIN ON THE BLOOD PROTEINS IN THE HORSE

Date	Amount of Toxin, C.c.	M. L. D.	Amount of Anti-toxin, Units	Tem-pera-ture	Total Protein, %	Total Albumin, %	Total Globulin. %
Jan. 11	...	...	...	....	7.4	3.7	3.7
Jan. 13	...	...	...	....	6.4	2.9	3.8
Jan. 17	...	...	...	....	6.2	2.9	3.3
Jan. 18	...	...	...	....	6.4	3.4	3
Jan. 21	...	200	...	....	5.8	3.6	2.2
Jan. 22	2						
Jan. 24	5						
Jan. 26	8	...	...	38.9			
Jan. 31	10						
Feb. 4	15	...	...	38.8			
Feb. 7	25	...	...	38.0			
Feb. 8	...	...	...	38.3	7.3	4	3.3
Feb. 11	40	...	...	37.9	...	...	...
Feb. 14	60	...	...	38.6	7.2	3.7	3.5
Feb. 18	90	...	...	39	7.5	2.5	5
Feb. 21	125	...	...	39.4			
Feb. 25	150	...	...	38.8	7.5	2.7	4.8
Feb. 28	175	...	...	38.9	8.2	2.3	5.9
Mar. 3	200	...	...	38.6	7.7	2.3	5.4
Mar. 6	250	...	...	39.5	7.7	2.6	5.1
Mar. 10	...	...	...	38.0	...	...	...
Mar. 14	...	...	800	38.6	...	...	...
Mar. 15	200	300	...	39			
Mar. 17	...	...	...	....	...	...	...
Mar. 20	250	...	...	38.8			
Mar. 24	300	...	...	38.6	...	...	...
Mar. 27	350	...	...	38.4			
Mar. 31	400	200	...	39.3	8.5	2.9	5.6
Apr. 3	450	200	...	39.0			
Apr. 7	500	...	...	38.9	...	...	...
Apr. 10	500	200	...	38.2			
Apr. 14	500	200	...	38.1			
Apr. 17	500	...	...	....	8.2	1.8	6.4
Apr. 21	...	...	...	37.9	4.5	1.2	3.3
Apr. 24	...	...	600	37.9	...	...	...
Apr. 25	400	200	...	39.2	8.1	0.3	7.8
Apr. 28	500	200	...	....	7.4	1.1	6.3
May 1	500	200	...	38.9	7.5	1	6.5
May 5	500	200	...	38.5	5.4	1	4.4
May 8	500	200	...	38.2	6.2	0.9	5.3
May 12	500	200	...	38.6			
May 15	500	200	...	38.6	7.9	1.5	6.4
May 19	...	...	...	38.2	8.6	2.2	6.4
May 23	...	...	500	38.1	7.6	0.2	7.4
May 24	500	...	...	38.4			
May 25	...	...	...	....	7.3	0.1	7.2
May 29	500	...	...	38.8	7.3	0.1	7.2
June 2	...	...	...	38.1	8	2	6
June 7	500	...	500	38.7	7.1	0.2	6.9
June 12	500	200	...	38.3	7.8	1.7	6.1
June 17	...	...	...	37.5	8.1	0.6	7.5
June 20	...	...	500	38.3	...	...	...
June 21	500	...	...	38.0	6.6	0.1	6.5
June 26	500	...	...	38.7	7.2	2.1	5.1
June 30	...	...	...	37.8	6.5	0.4	6.1
July 4	...	...	500	38.2	7.5	0.5	7

TABLE 3—Continued

EFFECT OF THE INJECTION OF DIPHTHERIA TOXIN ON THE BLOOD PROTEINS IN THE HORSE

Albumin of Total Protein, %	Globulin of Total Protein, %	Non-protein Constituents, %	Protein Quotient	Remarks
50	50	1.2	1	Bled 6 liters of blood
43	57	1.2	0.77	On Jan. 14, injection of 5,000,000 'antisuppurin'
46	54	1.2	0.8	
53	47	1.3	1.1	On Jan. 21, injection of 15,000 units of diphtheria antitoxin and 15,000 units of tetanus antitoxin
62	38	1.2	1.6	
56	44	1.1	1.2	
..	..	...	....	100 million antisuppurin
51	49	1.4	1	Bled before injection of toxin
33	67	1.3	0.5	100 million antisuppurin
36	64	1.5	0.56	1500 units of tetanus antitoxin injected
30	70	1.3	0.43	
30	70	1.3	0.42	100 million antisuppurin
33	67	1.4	0.5	
..	..	...	....	100 million antisuppurin
..	..	...	....	Bled 9 liters for plasma
..	..	...	....	100 million antisuppurin
..	..	...	....	1500 units tetanus antitoxin
34	66	1.1	0.51	
..	..	...	....	100 million antisuppurin
21	79	1.4	0.26	
26	74	1.1	0.36	1500 units tetanus antitoxin
..	..	...	....	Bled 9 liters for plasma
0.4	99.6	1.6	0.004	
1.5	98.5	1.4	0.015	
1.3	98.7	3	0.015	
1.8	98.2	5.8	0.02	1000 units tetanus antitoxin
1.7	98.3	4.7	0.02	
1.9	98.1	2.3	0.02	
25	75	1.5	0.3	500 units tetanus antitoxin
3	97	2.4	0.03	Bled 9 liters
2	98	1.3	0.02	
2	98	1.5	0.02	
25	75	1.4	0.33	1500 units tetanus antitoxin
3	97	1.3	0.03	Bled 9 liters
22	78	1.1	0.28	
8	92	1.2	0.08	1000 units tetanus antitoxin
..	..	...	....	Bled 9 liters
2	98	3.1	0.02	
30	70	1.5	0.4	
6	94	1.5	0.06	1000 units tetanus antitoxin
7	93	1.6	0.07	Bled 9 liters

TABLE 4

SERUM PROTEINS OF ANTITOXIC HORSE SERUM

Date	Name of Horse	Amount of Antitoxin, Units	Total Protein, %	Total Albumin, %	Total Globulin, %
		Dec. 7 400			
Dec. 20	Dolly		5.2	2.6	2.6
		Dec. 21 400			
Dec. 23	Dolly		5.1	2.4	2.7
Dec. 27	Dolly		5	1.5	3.5
Jan. 3	Dolly		6.6	3.5	3.1
		Jan. 4 400			
Jan. 12	Dolly		5.6	1.7	3.9
		Jan. 18 300			
Dec. 20	Daisy		5.9	2.6	3.3
		Dec. 21 500			
Dec. 23	Daisy		5.9	2.6	3.3
Dec. 27	Daisy		5.7	2.1	3.6
Jan. 3	Daisy		6.7	3	3.7
		Jan. 11 300			
Dec. 20	Maude	800	7.1	2.8	4.3
Dec. 23	Maude		7.3	3	4.3
Dec. 27	Maude		6.3	2.3	4
Jan. 3	Maude		8.5	3.1	5.4
		Jan. 11 700			
Jan. 12	Maude		7.9	2.2	5.7
Dec. 20	Anita	700	5.7	2.2	3.5
Dec. 23	Antia		6	3	3
Dec. 27	Anita		6	3.2	2.8
Jan. 3	Anita		5.8	2.7	3.1
Jan. 12	Anita		6.1	1.5	4.6
		Jan. 18 400			
		Dec. 7 300			
Dec. 20	Shag		4.5	1.6	2.9
Dec. 23	Shag		4.6	1.5	3.1
Dec. 27	Shag		4.36	1.36	3
		Dec. 28 300			
Jan. 3	Shag		3.7	1.5	2.2
Jan. 12	Shag		3.8	0.7	3.1
		Jan. 15 300			

TABLE 4—Continued

SERUM PROTEINS OF ANTITOXIC HORSE SERUM

Albumin of Total Protein, %	Globulin of Total Protein, %	Nonprotein Constituents, %	Protein Quotient	Remarks
				Dec. 3, 1914, started immunization
50	50	1.1	1	
47	53	1.1	1	Dec. 23, 1915, 500 c.c. toxin
30	70	1.3	0.4	Dec. 27, 1915, 500 c.c. toxin
56	44	1.3	1.2	
				Jan. 10, 1916, 500 c.c. toxin
30	70	1.3	0.4	
44	56	1.1	0.8	May 6, 1915, started immunization
				Dec. 13, 500 c.c. of toxin
44	56	1	0.8	Dec. 23, 500 c.c. of toxin
36	64	1.3	0.56	Dec. 27, 500 c.c. of toxin
				Dec. 31, 500 c.c. of toxin
45	55	1.3	0.9	
39	61	0.96	0.6	Sept. 21, 1915, started immunization
				Dec. 15, 500 c.c. of toxin
41	59	0.77	0.7	
				Dec. 24, 500 c.c. of toxin
36	64	1	0.56	Dec. 27, 500 c.c. of toxin
				Dec. 31, 500 c.c. of toxin
36	64	1	0.56	Jan. 3, 500 c.c. of toxin
27	73	1.1	0.37	Jan. 12, 500 c.c. of toxin
38	62	1.1	0.6	Dec. 8, 1914, started immunization
				Dec. 13, 300 c.c. of toxin
50	50	1	1	Dec. 23, 200 c.c. of toxin
53	47	1.3	1	Dec. 27, 250 c.c. of toxin
				Dec. 31, 250 c.c. of toxin
46	54	1.2	0.8	Jan. 3, 250 c.c. of toxin
				Jan. 7, 300 c.c. of toxin
24	76	1.5	0.3	Jan. 10, 300 c.c. of toxin
35	65	1	0.5	Jan. 20, 1915, immunization started
				Dec. 10, 100 c.c. of toxin
32	68	0.87	0.47	Dec. 13, 150 c.c. of toxin
				Dec. 17, 200 c.c. of toxin
32	68	1	0.47	Dec. 20, 200 c.c. of toxin
				Dec. 29, 150 c.c. of toxin
				Jan. 3, 200 c.c. of toxin
40	60	1.1	0.66	Jan. 7, 200 c.c. of toxin
20	80	1.5	0.25	
				(Animal pregnant)

The percentage of total proteins showed very strikingly fluctuations. Although certain periods were associated with definite increase in the amount (March 31 and May 19), this increase was not a constant feature during the course of immunization. In this respect, our observations do not agree with those of previous workers.[16]

The considerable increase in the nonprotein constituents of the serum which occurred between May 1 and May 8 are difficult to explain, unless they were the result of some metabolic upset, although there is no definite evidence for this surmise.

In Table 4 will be found additional evidence in support of some of the points brought out in the more complete experiment. This table contains some determinations made at random intervals in 5 horses at various stages of diphtheria immunization. A careful analysis of the results demonstrates clearly the contention already made that the height of immunity stands in no direct relationship to the degree of globulin rise. A few specific illustrations may be cited. The protein quotient in horse Dolly was found to be 1 on December 21, and 0.4 on January 18, although the number of antitoxic units differed only by 100 on the 2 dates. Again, with very little change in the quotient in horse Daisy on December 21, and January 11, a considerable difference in the degree of immunity on these 2 dates was found to be present.

B. *Immunization of the Goat.*—Complete studies were made on the immune bodies and the serum proteins in 2 animals over a period of 4 months (Table 5 and Chart 2). The general deductions permissable from these experiments do not differ essentially from those given in connection with similar observations in the horse. It will be necessary merely to call attention to several details peculiar to these experiments. In neither goat was an initial injection of diphtheria antitoxin given. Immunization was started with small doses (0.001 c.c.), and reached toward the end of the period of immunization a maximum of 32 c.c. (200-300 M. L. D.).

The very definite absence of parallelism between antibodies and globulins is well shown in the study of both animals: Mephistopheles and Gretchen. It will be seen, by referring to the table and charts, that in the former the highest grade of immunity was attained on October 23. At this time the globulins constituted 76% of the total proteins (protein quotient 0.3). But already, on September 23, a similar rise in globulins had been observed, at a time when the animal showed a very slight grade of immunity (2 antitoxic units). Attention should, furthermore, be directed to the interesting observation that, on November 4, a still greater depression of the quotient (globulin rise) was effected by the injection of 10 c.c. of toxin and 20 c.c. of a 10-day old culture of diphtheria bacillus, although the number of antitoxic units had already fallen from about 12 to 4.

Similar discrepancy may be noted in the case of the 2nd goat, Gretchen (Table 5). Here it will be noted that, on October 2, the globulins constituted 92% of the total proteins and that the immunity corresponded to 3 antitoxic units; whereas 1 month later the immunity had increased (6 antitoxic units), while the percentage of globulins had fallen (74%).

The behavior of the total proteins in both goats is similar in every respect to that noted in the horse. Whereas the process of immunization causes fluctuations in the total proteins, we have failed to note any definite and constant increase throughout the period of observation.

[16] Butjagin, P. W.: Hyg. Rundschau, 1902, 12, p. 1193. Joachim, J.: Arch. f. d. ges. Physiol., 1902, 93, p. 558.

C. *Immunization of the Rabbit.*—The immunization of rabbits with diphtheria or tetanus toxin is attended with great difficulties. This is more particularly true of diphtheria toxin. In this instance, successful immunizations can be carried out only if the toxin employed be attenuated. This was accomplished in our experiments by the addition of Lugol's solution to the toxin. In Table 6 and Chart 3, we have presented the detailed observations of 2 complete experiments with diphtheria toxin. In each experiment, the observations were extended over a period of 6 weeks, during which frequent determinations could be made of the degree of immunity developed and the concomitant alterations in the albumin-globulin ratio. These experiments illustrate in an unusual way the points which have already been emphasized in our experiments on the horse and goat, as well as in those previously recorded in connection with the influence exerted by bacterial toxins.[1]

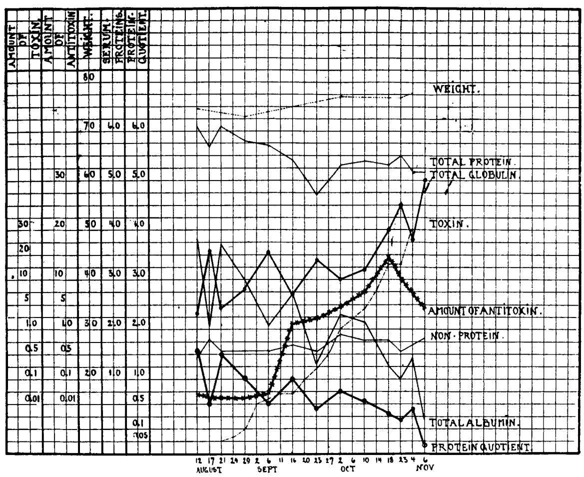


Chart 2.—Goat injected with diphtheria toxin.

The striking response of Rabbit 395 to inoculations of diphtheria toxin is well shown in Chart 3. It will be noted that, after the 2nd inoculation, an abrupt decline in the protein quotient (rise in globulins) occurred, and that this continued low until the end of the experiment, although the dose of toxin employed was not sufficient to develop within the animal any appreciable degree of antitoxic immunity. This animal possessed initially one fiftieth of an antitoxic unit which did not rise above one tenth of a unit during any period of the experiment. It must, therefore, be clear that the 2 responses of the animal are quite independent of one another, and that the inoculation of diphtheria toxin may produce this upset in the protein partition without at the same time giving rise to any appreciable grade of immunity.

The results of our observations on the 2nd animal (Rabbit 327) are essentially the same. These have been detailed in Table 6, and no additional comment is necessary.

TABLE 5

EFFECT OF THE INJECTION OF DIPHTHERIA TOXIN ON THE BLOOD PROTEINS IN THE GOAT "GRETCHEN"

Date	Amount of Toxin, C.c.	M. L. D.	Amount of Anti-toxin, Units	Weight, Pounds	Tempera-ture	Total Protein, %	Total Albumin, %
Aug. 12	....	....	1/50	63½	37.9	5.5	1
Aug. 17	....	....	1/50	....	37.8	5.8	3.2
Aug. 21	0.001	200	>1/50	64½	40.1	5.6	2.1
Aug. 24	0.002	200	......	....	39.1		
Aug. 29	0.005	200	>1/60	....	38.6	6.6	3.3
Sept. 2	0.01	200	......	....	39.5	...	...
Sept. 6	0.02	200	1/50	63¾	39.3	6.2	2.3
Sept. 11	0.04	200	......	....	38.7		
Sept. 16	0.04	1.+0.54 >200	½-1	....	38.6	5.9	2
Sept. 20	0.1	200	......	....	38.0		
Sept. 23	0.2	L+0.29	2	....	38.6	5.7	1
Sept. 27	0.5	L+0.29	......	....	38.5		
Oct. 2	1	L+0.425	3	....	38.9	5.4	0.4
Oct. 6	2	L+0.425	......	....	38.5		
Oct. 10	4	200	3-4	....	39	5.5	1
Oct. 14	8	L+0.54	......	....	39.3		
Oct. 18	16	200	4	....	39.5	5.7	2.1
Oct. 23	32	<100	3-4	61	37.8	5.8	1.7
Oct. 30	....	....	......	....	39.1	...	...
Nov. 4	8	300	6	57½	38.3	5.4	1.4
Nov. 6	....	....	5	....	37.9	5.2	0.1

TABLE 6

EFFECT OF THE INJECTION OF DIPHTHERIA TOXIN ON THE BLOOD PROTEINS IN THE RABBIT 327

Date	Amount of Toxin, C.c.	M. L. D.	Amount of Anti-toxin, Units	Weight, Gm.	Total Protein, %	Total Albumin, %	Total Globulin, %
Aug. 31	..	.....	<1/100	2900	5.3	4	1.3
Sept. 6	..	.....	<1/100	2850	5.5	3.5	2
Sept. 19	3	<100	<1/100	2800	5.8	3.2	2.6
Sept. 21	..	.....	<1/100	2700	5.7	2.7	3
Sept. 25	5	<100	1/100	2750	5.5	2	3.5
Sept. 28	..	.....	1/75-1/100	2675	5.8	2.6	3.2
Oct. 2	12	<100	1/75-1/100	2725	5.4	1.1	4.3
Oct. 9	..	.....	<1/75	2675	5.9	3.2	2.7
Oct. 11	5	200	1/75	2800			
Oct. 12	..	.....	1/50-1/75	2700	5.2	0.7	4.5

TABLE 5—*Continued*

EFEECT OF THE INJECTION OF DIPHTHERIA TOXIN ON THE BLOOD PROTEINS IN THE GOAT "GRETCHEN"

Total Globulin, %	Albumin of Total Protein, %	Globulin of Total Protein, %	Nonprotein Constituents, %	Protein Quotient	Remarks
4.5	20	80	1.4	0.25	
2.6	55	45	1.6	1.2	
3,5	40	60	1.6	0.6	No reaction
3.3	50	50	1.4	1	
...	..	..	...	...	Indigestion as a result of atony of the rumen
3.9	37	63	1.4	0.6	
3.9	34	66	1.4	0.51	
4.7	17	83	1.3	0.2	
5	8	92	1.5	0.08	Animal was pregnant
4.5	19	81	1.4	0.23	
3.6	36	64	1.5	0.56	
4.1	30	70	1.3	0.43	
...	..	..	...	....	Aborted 2 fetuses about 8-10 weeks old
4	26	74	1.5	0 35	Toxin was mixed with 30 c.c. of a 10-day culture of the diphtheria bacillus, Park and Williams, in Martin's broth
5.1	2	98	1.6		

TABLE 6—Continued

FFECT OF THE INJECTION OF DIPHTHERIA TOXIN ON THE BLOOD PROTEINS IN THE RABBIT 327

Albumin of Total Protein, %	Globulin of Total Protein, %	Nonprotein Constituents, %	Protein Quotient	Remarks
75	25	2.1	3	
64	36	1.4	1.8	
55	45	1.4	1.2	Diphtheria toxin mixed with 1 c.c. of Lugol's solution, and kept at room temperature for 15 minutes
47	53	1.6	0.88	
34	66	1.8	0.5	Diphtheria toxin mixed with 2 c.c. of Lugol's solution; blood pressure low
44	56	1.6	0.8	
20	80	1.6	0.25	Diphtheria toxin mixed with 3 c.c. of Lugol's solution
54	46	1.5	1.1	
14	86	1.9	0.16	Died during the afternoon; hemorrhagic gastro-enteritis; focal necrosis of the liver; hemorrhages in the mesentery and cortex of suprarenals; acute nephritis

D. *Immunization of the Dog.*—In the description of the methods employed in our study, brief mention was made of the great susceptibility of the dog to toxin inoculations. Such inoculations frequently result in necrosis and sloughing at the seat of injection. In a previous paper, it has been shown that such secondary infections, in themselves, will produce a striking upset in the partition of the protein fractions, which at times may amount to a complete inversion of the formula. To overcome this complication, several modifications of the technic used in the case of the other animals experimented on were tried. An attempt was first made to lessen the initial response of the animal by the inoculation of balanced mixtures of diphtheria toxin and antitoxin. But such mixtures were not found to give the desired effect. Necroses and sloughs still resulted. Better success was obtained by initial inoculations of diphtheria antitoxin. As soon as a basic immunity was established, hyperimmunization could be accomplished without the production of sloughs at the site of inoculation.

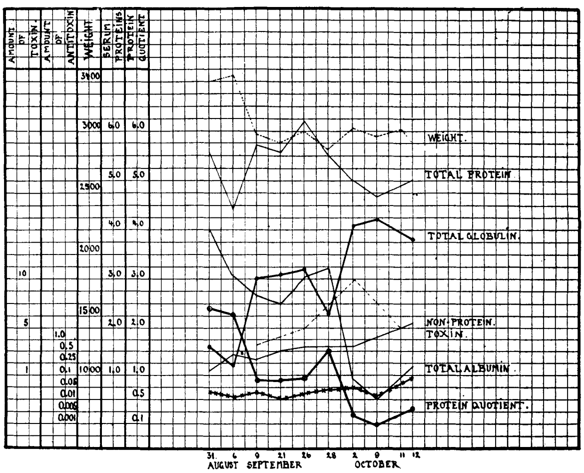


Chart 3.—Rabbit injected with diphtheria toxin.

Of the 6 dogs experimented on, we wish to present the results obtained in 2 animals only, because these are more complete and are representative of all the others. The detailed observations are given in Table 7 (Dog 641) and Chart 4 (Dog 640). But for purposes of clearness, it may be well to call attention to a few of the interesting features of these experiments.

The immediate effect which an inoculation of toxin may have on the albumin-globulin ratio is well shown in Table 7 (Dog 641). In this animal, notwithstanding the protective injection of 1 large dose of antitoxin, great fluctuations in the protein quotient were observed, due to the immediate rise in the globulins following the repeated inoculations. Such periods of globulin increase, as can be readily seen from the table, were not attended by a corresponding rise in the number of antitoxic units. These continued low for 2 months after the beginning of the experiment, when they increased to a level of 14-15 units, and then showed a tendency to decline, due to the lower toxicity of the prepa-

ration employed at this time.* The figures of the table show clearly that periods of increased immunity do not parallel periods associated with an increase in the globulin content. Thus, on July 1 and 24, this animal showed a globulin content of about 92-93%, with an antibody content of 1/5-1/2 of an antitoxic unit, whereas, on August 19, with a maximum number of antitoxic units 14-15, the globulin percentage only reached 53. A similar lack of parallelism will be seen in the results of Dog 640, graphically presented in Chart 4. This shows in an unusual way the gradual rise in the antibody curve without a parallel rise in the curve of the protein quotient. Such fluctuations as were noted are for the most part transitory. The failure to obtain any higher grade of immunity, despite the increase in the dosage of the toxin, must be attributed to the low toxicity of the preparation available at this time.

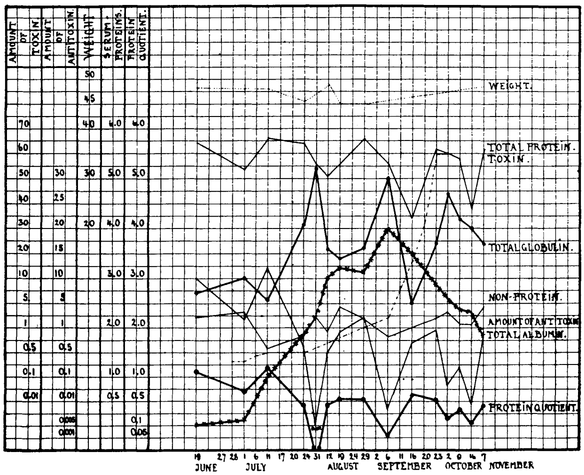


Chart 4.—Dog injected with diphtheria toxin.

The chart shows, furthermore, 2 additional features worthy of emphasis. It will be noted, in the first place, that the increase in globulins occurs, for the most part, at the expense of the albumin fraction, so that the latter curve is almost a mirror-image of the former, a relationship of the 2 fractions which has also been noted in bacterial infection and immunity; secondly, attention should be called to the considerable fluctuations in the total proteins of the blood serum without any constant and persistent increase in their percentage during the entire period of immunization.

II. IMMUNIZATION WITH TETANUS TOXIN

The literature contains only an occasional observation of the influence of tetanus toxin on the blood proteins. Because of the interesting results we obtained by the use of diphtheria toxin, we thought it worth while to extend our studies in this direction, although our experiments

* Martin L. (Ann. de l'Inst. Pasteur, 1898, 12, p. 26) has shown that a low antitoxic serum will result, following the injection of a weak subsequent to inoculations of a strong one.

TABLE 7

EFFECT OF THE INJECTION OF DIPHTHERIA TOXIN ON THE BLOOD PROTEINS IN THE DOG

DOG 641

Date	Amount of Toxin, C.c.	M. L. D.	Amount of Antitoxin, Units	Weight, Pounds	Temperature	Total Protein, %	Total Albumin, %
June 19	...	...	>1/150	40½	38.2	5.7	2.5
June 27	...	...	......	39½	38.8	...	...
June 28	0.3	200	......	....	39.1	...	...
July 1	0.3	200	1/20	....	38.6	5.2	0.43
July 6	0.3	200	......	....	38.3		
July 11	0.5	200	1/5	....	39.0	6.2	3.2
July 17	0.6	200	......	....	38.3		
July 20	...	...	......	42	39.6	...	...
July 24	...	...	1/2	40½	38.3	6	0.5
July 31	...	...	1–2	....	38.1	5.9	1.3
Aug. 12	0.7	200	......	44½	38.2	5.5	2.5
Aug. 19	1	200	14–15	42½	38.1	5.5	2.6
Aug. 24	1.5	200	......	41¾	38.3		
Aug. 29	2.5	200	12	43	38.3	5.9	2.3
Sept. 2	5	150–200	......	....	37.9		
Sept. 6	10	200	10	....	39.0	5.4	0.4
Sept. 11	20	<100	......	....	38.1		
Sept. 16	40	<100	15	....	38.2	6.5	3.5
Sept. 20	80	<100					
Sept. 23	....	...	10	....	....	6	2.1
Oct. 2	30	<200	6–8	43	38.2	5.1	0.1
Oct. 9	....	...	6	....	38.1	6	4
Oct. 16	....	...	5	....	38.1	5.7	0.9
Nov. 7	....	...	2	43¾	....	5.6	1.4

TABLE 8

SERUM PROTEINS OF ANTITOXIC HORSE SERUM

TETANUS

Date	Number of Horse	Amount of Antitoxin, Units	Total Protein, %	Total Albumin, %	Total Globulin, %
Jan. 17	12 (Op. 216)	500	7.3	2.7	4.6
Jan. 17	13 (Op. 217)	200	5.9	2.3	3.6
Jan. 23	15 (Op. 218)	125	5.7	2.7	3
Feb. 7	(Op. 223)	160	5.7	1.8	3.9
Feb. 7	(Op. 224)	602	7.2	2.7	4.5
Feb. 7	(Op. 225)	250	6.9	3	3.9
Feb. 7	(Op. 226)	400	5.9	2.3	3.6
Feb. 7	(Op. 227)	200	5.7	2.3	3.4
Feb. 10	(Op. 228)	909	7.9	2.8	5.1

TABLE 7—*Continued*

EFFECT OF THE INJECTION OF DIPHTHERIA TOXIN ON THE BLOOD PROTEINS IN THE DOG 641

Total Globulin, %	Albumin of Total Protein, %	Globulin of Total Protein, %	Nonprotein Constituents, %	Protein Quotients	Remarks
3.2	50	50	2.6	1	
...	..	..	...	...	6000 units diphtheria antitoxin subcutaneously
...	..	..	...	...	No clinical symptoms
4.8	7	93	2.3	0.07	No symptoms
3	51	49	1.8	1	Swelling at seat of injection
...	..	..	...	...	Slight intoxication resulting from accidental feeding of liver tissue; considerable swelling at seat of last injection; warm and tender
5.5	8	92	1.6	0.08	At seat of last inoculation, a dark, necrotic, dry slough 5½ × 5 cm. diameter
4.6	22	78	2.2	6.3	Necrotic area well demarcated; removed slough, and treated granulated wound with methylene blue
3	45	55	1.9	0.8	Wound 1½ × 1½ cm. diameter was granulating well
2.9	47	53	2.3	0.88	At seat of last injection, swelling size of a hazel nut
3.6	40	60	2	0.6	
5	6	94	2	0.06	
3	46	54	2.1	0.8	
3.9	35	65	2	0.5	
5	2	98	2	0.02	
2	60	40	2	1.5	
4.8	16	84	1.9	0.2	
4.2	25	75	2	0.3	

TABLE 8—*Continued*

SERUM PROTEINS OF ANTITOXIC HORSE SERUM

TETANUS

Albumin of Total Protein, %	Globulin of Total Protein, %	Nonprotein Constituents, %	Protein Quotient	Remarks
37	63	1.2	0.6	
39	61	1.4	0.65	Maximum unitage
47	53	1.4	0.88	
57	69	1.4	0.45	Decline of antitoxin production
37	63	1.4	0.6	Decline of antitoxin production
43	57	1.3	0.75	Maximum unitage
39	61	1.5	0.6	
40	60	1.4	0.66	
54	46	1.1	1.1	Maximum unitage

with the use of tetanus toxin are not so complete. Besides a series of determinations on the albumin-globulin ratio of 9 horses studied during various stages of tetanus immunization, we have carried out a number of systematic experiments on rabbits. The general methods employed are analogous to those described in connection with the use of diphtheria toxin. The use of so sensitive an animal as the rabbit necessitated an attenuation of the toxin with Lugol's solution and a careful grading of dosage, so as to make a more prolonged study of the animals possible. In the beginning of this series, we failed to attenuate the toxin sufficiently so that the animals succumbed after a short period with symptoms of tetanus intoxication. The intervention of such symptoms, although not anticipated, proved to be of extreme interest,

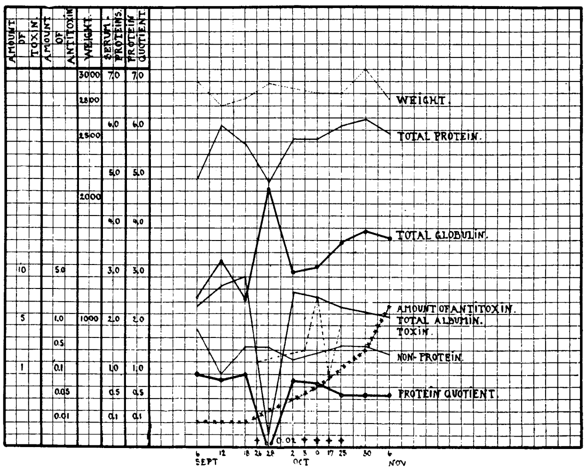


Chart 5.—Rabbit injected with tetanus toxin.

for as will appear (Table 10), this mishap threw some light on the behavior of the serum proteins during the development of this particular intoxication.

In Table 8, we have recorded the results of determinations made on 9 horses, at varying intervals, during immunization with tetanus toxin. The figures support in an unusual way the conclusions to which we have arrived from previous experiments. At the time the serum proteins were quantified, we as yet had no definite knowledge of the degree of immunity present in the animals studied. A comparison of the figures obtained for the antitoxic units with those for the globulin content permit of no doubt concerning the justness of our contention that there can be no relationship between the 2 phenomena.

A few instances in support of this fact will suffice. In the table it will be noted that the horse (operation-specimen No. 228) which developed the highest grade of immunity (909 antitoxic units) showed on the day the proteins were studied a globulin percentage of 46; whereas, in the horse (Op. 218) which developed the lowest immunity of this series, we observed a slightly higher globulin content.

In order to obtain additional evidence for or against this deduction, however, we carried out 2 complete immunization experiments on rabbits (Table 9 and Chart 5). Reference to the general procedure used has been made, and attention need only be directed to a few of the detailed results. These findings (Chart 5) are presented in a very striking manner for 1 of the animals (Rabbit 714). Only at 1 period during the experiment did the globulin fraction rise precipitously (fall in protein quotient). This occurred on September 28, following the injection of 1.5 c.c. of tetanus toxin. Four days later the quotient showed a tendency to return to a normal level and the curve now continued along a parallel course despite the very definite increase in this animal's immune state, as indicated by the great rise in the number of antitoxic units (2-5 units). A similar result was obtained in the 2nd animal (Rabbit 715, Table 9), but, in this instance, the striking rise in the globulin percentage was somewhat retarded, and occurred only after the 2nd and 3rd injections of tetanus toxin. Notwithstanding the gradual increase in the animal's immunity, the protein quotient tended to return to its initial normal level. Such an observation has already been recorded in connection with some experiments previously published on the effect of bacterial toxins on changes in the blood protein fractions.

Interesting in this connection are the 2 observations which we made on the influence of tetanus intoxication on the blood proteins. As previously stated, these results were quite incidental and not anticipated, but they afford data of great comparative value. The attenuation of the toxin was apparently not sufficient to prevent the appearance of toxic symptoms, for both animals died on the 7th day with typical signs of tetanus: opisthotonus trismus, salivation, and clonic and tonic spasms. In spite of this mode of death, no change occurred in the albumin-globulin ratio. It is somewhat difficult to harmonize this finding with those previously recorded in other forms of intoxication. It has been found, for instance, that animals developing toxic symptoms after overwhelming inoculations of bacterial toxins[1] or following the absorption of loop proteose[17] invariably show an increase in the globulin content. The animals dying of tetanus intoxication, however, showed no alteration in the quotient. The only possible explanation for this apparent discrepancy, it seems to us, must be sought in the nature of the tetanus toxin. The latter is, as we know, primarily a neurotoxin, with an almost specific affinity for nervous tissue; whereas the other toxic substances produce very striking alterations in body tissues in general. Since it has become clear that alterations in the albumin-globulin ratio stand in intimate relationship to a general metabolic disturbance associated with much tissue destruction, it is not surprising to find an absence of fluctuations in the protein quotient during the action of a substance possessing, for the most part, a neurotoxic action.

[17] Hurwitz, S. H., and Whipple, G. H.: Jour. Exper. Med., 1917, 25, p. 231.

III. IMMUNIZATION WITH BOTULISM TOXIN

Through the kindness of Dr. E. C. Dickson, some potent toxin of B. botulinus was placed at our disposal. The study of immune reactions with this soluble toxin appealed to us as of especial interest from a comparative viewpoint, because it has been established that the local and constitutional reaction following the injection of this toxin differ in many important respects from those met with after inoculations of diphtheria and tetanus toxin. Unlike the latter 2 toxins, botulism toxin does not produce so marked a constitutional and temperature reaction, and in other respects is quite different in its manifestations. Because of these points of difference, it seemed worth while to determin in what manner inoculations of the toxin of botulism affected the partition of the blood proteins, and how these alterations were related to the grade of immunity developed.

TABLE 9

EFFECT OF THE INJECTION OF TETANUS TOXIN ON THE BLOOD PROTEINS IN THE RABBIT

Date	Amount of Toxin, C.c.	M. L. D.	Amount of Antitoxin, Units	Weight, Gm.	Total Protein, %	Total Albumin, %	Total Globulin, %
Sept. 13	..	...	$<1/20$	2525	5.7	3.9	1.8
Sept. 18	..	...	$<1/20$	2625	5.7	3.8	1.9
Sept. 26	1	100	......	2600	...	...	...
Sept. 28	..	...	......	2700	5.6	3.1	2.5
Oct. 2	..	...	$<1/20$	2700	6	4.2	1.8
Oct. 3	5	100	......	2675	...	...	...
Oct. 9	12	100	$<1/10$	2600	5.7	2	3.7
Oct. 16	..	...	$<1/10$	2625	6.2	1	5.2
Oct. 17	1	1000	......	2700			
Oct. 23	5	1000	$>1/5$	2520	6.1	2.8	3.3
Oct. 31	..	...	$>1/5$	2850	6.1	2.8	3.3
Nov. 6	..	...	1+	2660	6.5	4.3	2.2

Our experiments were carried out on dogs. It is commonly stated that this animal is less susceptible to botulism toxin than are the smaller laboratory animals, rabbits and guinea-pigs. But we have found in confirmation of the work of Dickson that this is not always the case, for the sample of toxin employed by us proved to be quite potent in its action, as evidenced by the death of 1 of the animals experimented on, and by the development of a fairly high grade of immunity in the 2nd dog. Because of the uniqueness of the observations with this toxin, a more detailed citation of the experiments carried out may be of interest.

EXPERIMENT 1.—*Death from Botulism Toxin.*—A black mongrel male weighing 35 pounds was inoculated subcutaneously on December 20, with 0.05 c.c. of toxin. No reaction occurred. On December 22, 0.1 c.c. toxin was inoculated. This was followed by the injection of 0.2 c.c. toxin on December 26, and 0.5 c.c. on December 30. Two days later the animal was prostrate. There was salivation, constipation, conjunctivitis, paresis of the hind legs, ataxia, and pharyngeal palsy. The animal vomited frothy bile and mucus, showed excessive salivation and lacrimation, and died at 1:30 p. m.

Autopsy.—The abdominal cavity was dry. A small amount of blood-stained fluid was found in both pleural cavities, a few drops of fluid in the pericardial sac.

The spleen was of normal size, but showed soft dark infarct. The kidneys were normal in size and appearance.

The jejunum was dry and revealed bile-stained contents. The ileum, colon, and rectum were very dry, and revealed poorly digested contents.

TABLE 9—*Continued*

EFFECT OF THE INJECTION OF TETANUS TOXIN ON THE BLOOD PROTEINS IN THE RABBIT

Albumin of Total Protein, %	Globulin of Total Protein, %	Nonprotein Constituents, %	Protein Quotient	Remarks
68	32	1.7	2.1	
66	34	1.6	1.9	
..	..	...	...	2 c.c. of tetanus toxin mixed with 2 c.c. of Lugol's solution, and kept at room temperature for 45 minutes
55	45	1.9	1.2	
70	30	1.6	2.3	
..	..	...	...	5 c.c. of toxin mixed with 2 c.c. of Lugol's solution
35	65	1.4	0.5	12 c.c. of tetanus toxin mixed with 2 c.c. of Lugol's solution
17	83	1.6	0.2	
45	55	1.4	0.8	
45	55	1.4	0.8	
66	44	1.2	1.6	

The liver was enlarged, engorged with blood, and revealed a granular surface. The gallbladder was distended with deep greenish-yellow bile. The mucous membrane was covered with a thick layer of mucin; numerous petechiae were observed.

The stomach contained frothy, bile-stained mucus. The mucous membrane was uniformly bile-colored. The fundus region was deep red.

The heart was flabby; the endocardium and valves were normal.

The bronchial mediastinal lymph nodes were enlarged.

The lung revealed a small thrombus in the pulmonary artery, and an infarct surrounded by compensatory emphysema. The middle lobe was pneumonic, and showed fresh fibrinous exudate on the pleura. In the right principal lobe, there was an infarct the size of a large pea; numerous similar infarcts were found in the left anterior heart lobe and principal lobe. The trachea and bronchi contained blood-stained froth.

The base of the brain was distinctly injected, as was also the dura of the spinal cord.

Microscopic study showed multiple thrombi in lung, spleen, and base of brain. The fluctuations which occurred in the serum-protein fractions during the course of immunization and the number of antitoxic units produced are presented in detail in Table 10.

EXPERIMENT 2.—*Successful Immunization with Botulism Toxin.*—A mongrel male dog weighing 40½ pounds was inoculated on January 18, with 0.1 c.c. of toxin (M. L. D. 2000). Two subsequent inoculations of 0.2 c.c. of toxin were given on January 25 and 30. On February 5, the animal showed a slight conjunctivitis and rhinitis. Three days later (February 8), animal received 0.5 c.c. toxin (M. L. D. 1000) and on February 14, 0.8 c.c. of the same toxin was given. On February 20, animal received 1 c.c. of toxin (M. L. D. 500), twice

TABLE 10

EFFECT OF THE INJECTION OF BOTULISM TOXIN ON THE BLOOD PROTEINS IN THE DOG

Date	Amount of Toxin, C.c.	M. L. D.	Amount of Antitoxin, Units	Weight, Pounds	Temperature	Total Protein, %	Total Albumin, %
Dec. 18	....	....	<1/20	35	39.9	3.9	2.1
Dec. 19	....	....	......	..	38.1	4.8	2.8
Dec. 20	0.05	2000	......	..	39.4	5.3	2.8
Dec. 22	0.1	2000	......	35	39.1	5.3	3.3
Dec. 26	0.2	2000	......	..	39.3	5.3	3.6
Dec. 28	...	....	......	..	37.8	5.4	3.8
Dec. 30	0.5	2000	......	..	38.1	5.4	2.3
Jan. 2	...	....	......	31.5	39.1	5.1	2.8
Jan. 4	...	....	<1/20	31.5	38.7	5.8	3.2
Jan. 5	...	....	......	31	40.5	5.9	3.3

this dose 4 days later, and 4 c.c. of the same toxin 8 days later. Two c.c. of a stronger toxin (M. L. D. 2000) was administered on March 7. On March 9, animal was found sick, and showed a subnormal temperature. The next 2 injections of 4 c.c. and 8 c.c., on March 14 and 17, respectively, were well tolerated. On March 26, a final injection of 15 c.c. of toxin of a strength greater than 2000 M. L. D. produced vomiting and constipation on the day following. With the exception of a moderate loss in weight during the period of immunization, the animal showed no other untoward symptoms, beside those already mentioned.

Daily specimens of blood were obtained for a determination of the degree of immunity present and for a quantification of the serum proteins. The interesting changes which this dog showed in the albumin-globulin ratio following the toxin injections are graphically presented in Chart 6.

The most striking feature of both the experiments with botulism toxin, and more especially of the latter, is the character of the immediate response of the animal to the inoculations. Unlike the reaction observed for other toxins, botulism toxin causes a depression of the

globulin content, with a corresponding rise in the albumin fraction. In Experiment 2, this first becomes noticeable after the 3rd inoculation (January 30), when the globulins begin to fluctuate gradually downward. The protein quotient shows marked fluctuations with a tendency to rise tremendously (fall in globulins) about 24-48 hours after each inoculation, reaching its maximum height about 2 months after the onset of the experiment. Only after the 3 last inoculations of massive doses of a strong toxin did the protein quotient tend to remain more or less permanently depressed. Careful analysis of the curve clearly

TABLE 10—*Continued*

EFFECT OF THE INJECTION OF BOTULISM TOXIN ON THE BLOOD PROTEINS IN THE DOG

Total Globulin, %	Albumin of Total Protein, %	Globulin of Total Protein, %	Nonprotein Constituents, %	Protein Quotients	Remarks
1.8	55	45	2.6	1.2	By mistake vaccinated against distemper on Dec. 17
2	58	42	2	1.3	
2.5	53	47	1.8	1.1	
2	63	37	1.5	1.7	
1.7	68	32	1.4	2.1	
1.6	71	29	1.4	2.4	
3.1	43	57	1.3	0.75	
2.3	55	45	2.7	1.2	Dog unable to rise, constipated, salivation, and conjunctivitis, pupils wide open, posterior paresis
2.6	56	44	2.2	1.2	Spastic posterior paresis, pharynx paralysis, accelerated pulse, 126; signs of ataxia
2.6	56	44	2.3	1.2	Vomited frothy bile and mucus, excessive salivation and lacrimation; died at 1:30 p. m., blood collected at 10 a. m. Necropsy: thrombosis of pulmonary arteries and veins

shows that the increase in the albumin fraction occurred at the expense of the globulin fraction, for the 2 curves are almost mirror-images of one another.

The total proteins fluctuated considerably during the experiment, and at times showed a slight tendency to rise, but no definite and persistent increase was noted for the whole period of immunization.

DISCUSSION

The studies of a number of workers and our own have shown with considerable certainty that an increase in serum globulins occurs with great constancy at one period or another during the immunization of an animal with a soluble toxin. But the question of the possible significance of this increase in relation to immunity and to the chemical nature of the protective substances formed has called forth numerous

studies and conflicting deductions. The evidence gained from our experiments with soluble toxins supports the contentions which we presented in a previous paper on the relation between blood globulins and bacterial infection and immunity. In general, the observations permit of the conclusion that no constant relation exists between the extent of serum globulin increase and the antitoxic content of the serum. The reasons for this deduction have been discussed in some detail in connection with the citation of the separate experiments, and for this reason, we believe that any additional comment is not needed. It may be more desirable to touch on the possible influence which certain experimental factors may have in giving rise to this increase in the globulins and to present certain tentative suggestions concerning other causes which may explain the observations presented.

The use of a variety of animals, as well as of a number of different toxins, has brought out several interesting points relative to animal susceptibility and the response to the toxin inoculations. It has been found, for instance, that in the less susceptible animal, the toxin must reach a certain threshold before a marked and constant depression of the protein quotient occurs. Thus, in the horse and goat, immunization must be continued over a longer period of time or with more potent toxins before any appreciable changes are produced; whereas in the dog small amounts of some of the toxins will rapidly bring about a striking alteration in the albumin-globulin ratio. Thus it would seem that something in the nature of a cumulative action or a summation of effects is needed to explain the delayed reaction observed in the less susceptible animals. Some such inference may serve also to explain the very unique observations made in connection with botulism toxin. It will be recalled that this toxin evokes a rise in the albumin fraction instead of the usual increase in the globulin fraction which may not occur until late in the experiment. The meagerness of our knowledge concerning the nature of botulism toxin does not justify extensive speculation, but it may not be too far afield to entertain the possibility that this toxin contains more than 1 toxic substance, and that 1 of these gives rise to an increase in the protein quotient; whereas the other causes a depression of it. Inasmuch as a depression of the quotient occurs after the inoculation of the majority of toxins, we may assume that the cause of this is attributable to its nonspecific component, which must reach a certain concentration before it becomes effective. On the other hand, the immediate and constant rise in the albumin fraction following the injections even of small doses of botulism toxin favors the assumption that this is due to the activity of

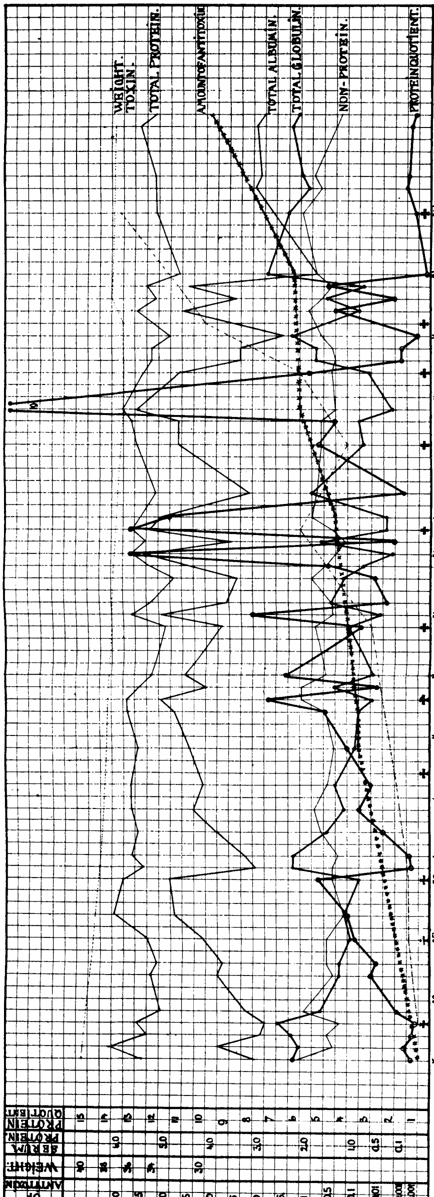


Chart 6.—Dog injected with botulism toxin.

a very active specific component which behaves in this characteristic way. Such speculation may gain some support from the study of other toxins which behave in this peculiar way. Thus far, however, this is an isolated observation.

It may be further noted that the animals inoculated with botulism toxin showed no constitutional reaction and responded by the production of antibodies in high concentration, although this response was associated in the beginning with a rapid rise in the albumin fraction. This is in striking contrast to the findings in those animals which reacted severely to the inoculations. As has been pointed out in another connection, such animals usually show a high globulin content. It has been shown by von Ermengen that the dog is not very susceptible to inoculation of botulism toxin, but Dickson has shown that the toxic substances produced by the California strain may be fatal in comparatively small doses, when inoculated into the dog.

From a large number of observations, we have become convinced that the serum globulin increase does not represent the accumulation of protective substances as Mellanby[18] and others have assumed. It would appear rather that other factors are responsible for the changes in the protein partition observed.

Excessive bleedings and the injection of large amounts of culture mediums are in themselves not responsible for the alteration in the albumin-globulin ratio.

To overcome the possible disturbing influence of excessive bleedings for serum-production, Gibson and Banzhaf bled their animals only after maximum antitoxic and protein changes had occurred, so they conclude that the variations observed during the period of increasing antitoxic potency are not influenced by severe hemorrhage. Furthermore, the possible influence of this factor in the production of the changes noted can be excluded in the smaller animals like the dog or rabbit, where the individual bleedings were small (10 c.c. for dog and 5 c.c. for rabbit).

Still another factor has been suggested as the possible cause of the rise in globulins, namely, the injection of large quantities of culture mediums together with the toxin. This point has been especially studied by Ledingham[6] during the immunization of the horse with diphtheria toxin. This worker was able to show conclusively that the inoculation of as large a quantity as 800 c.c. of bouillon into a susceptible horse did not produce any appreciable change in the globulin

[18] Proc. Royal Soc., Series B, 1908, 70, p. 399.

percentage. Our own studies have shown that the injection of about 10 c.c. of bouillon into the dog gives rise to only normal fluctuations in the protein fractions, whereas a similar quantity of broth-containing toxin causes a very striking alteration of the ratio. It is, therefore, unlikely that either excessive hemorrhage or the injection of large quantities of culture medium can explain the changes observed.

Whatever the ultimate cause or causes of these phenomena may be, there is good reason to believe that the upset in the delicate protein balance of the blood is 1 manifestation of a disturbed metabolism resulting from the toxin inoculations. The reasons for assuming the existence of a metabolic disorder in the animals studied have been given and discussed in previous papers. At that time we ventured the suggestion that the heaping up of blood globulins might be the result of the more rapid formation of this molecule. We have come to believe, however, that such speculation is not permissible at the present time because of the incompleteness of our knowledge concerning the chemical relationship of the albumin and globulin fractions. It is fair to state, however, that the work[19] which has been done thus far strongly suggests that the conversion of the one fraction into the other, or the change of one form of globulin into another is brought about by an alteration of the physico-chemical properties of the blood serum, properties on which, in all probability, depend the differentiation of the various protein fractions by "salting out methods." It may, therefore, not be too venturesome to suggest that the alteration in the normal albumin-globulin ratio which has been observed during immunization may be attributable to change in the colloidal properties of the serum, which may in great measure determine its reaction to the various precipitating salts.

CONCLUSIONS

The percentage of serum globulins increases markedly during the course of immunization with diphtheria, tetanus, and botulism toxin. In the case of botulism toxin, however, there is first an initial rise in the albumin fraction.

No constant relationship is demonstrable between the percentage increase in the serum globulin and the antitoxic potency of the serum.

The rise in globulins may be 1 manifestation of an upset in the delicate protein balance of the blood, resulting from the disturbed metabolism following the toxin inoculations.

[19] Berg, W. N.: Jour. Agric. Research, 1917, 8, p. 449.